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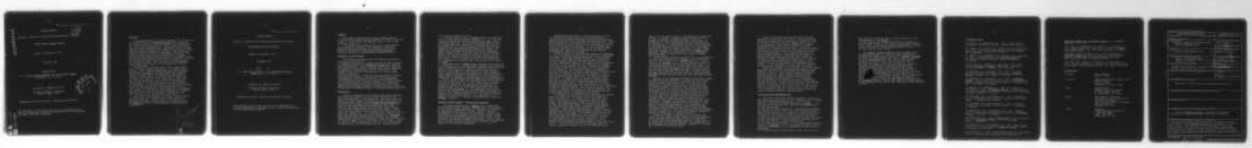

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REPORT NUMBER 8

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# Malaria: Biology of the Merozoite-Erythrocyte Interface

SECOND ANNUAL SUMMARY REPORT

Robert O. McAlister, Ph.D.

December 1976

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D. C. 20315

Contract No. DAMD17-75-C-5037

Southern Methodist University
Dallas, Texas 75275



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Abstract:

Detailed evaluation of several methods for the isolation of free merozoites of Plasmodium berghei was conducted. All methods were found to yield preparations contaminated by a small but finite number of intact parasitized cells. The free parasite preparations, irrespective of the means used for their isolation, were found to be unable to invade susceptible host erythrocytes in vitro, under conditions known to support invasion of merozoites originating from schizonts which had ruptured spontaneously in vitro. The free parasite preparations isolated using either continuous-flow ultrasound or multiple-burst ultrasound, followed by differential centrifugation, were evaluated in vivo for invasive ability. All infectivity of these preparations could be accounted for as contaminating intact parasitized cells.

Studies were performed using a means unequivocally known to yield invasive merozoites, i.e. immune lysis in vivo. Free parasite preparations were also studied after lysis in vitro using this method; once rendered free of the host cells, the merozoites were found to undergo a rapid loss in invasive ability when held in a diluent not found toxic to intact cells. This loss of function occurred at 1-2$^{o}$C, and was exacerbated by washing the parasitized cells prior to sensitization with antibody. It was concluded that previous efforts to demonstrate invasive ability by free parasites isolated using various methods were unsuccessful due to this rapid loss of invasiveness. Free parasites of P. berghei are impossible to study from the standpoint of invasive ability, unless they are exposed to susceptible host cells within 15 min following host cell lysis.

The results are discussed in regard to the findings of others with this species, and to other species of malarial parasites. Future studies will involve the adaptation of newly discovered culture methodology for P. falciparum to one or more species of rodent malarial parasites.

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FOREWORD

This report constitutes an annual progress summary of work performed under Contract #DAMD17-75-C-5037, initiated 1 Apr 75. The Contract was renewed 1 Apr 76, and is due to expire 31 Dec 76.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee of the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

Statement of the Problem:

The Contract was awarded for the purpose of pursuing the following objectives:
(1) Detailed evaluation of several methods for the isolation of invasive merozoites of Plasmodium berghei from infected mouse erythrocytes. Use of biological, physical, chemical, and mechanical means would be tested under direct comparison, to determine the method best for the isolation of large numbers of this stage of the parasite in a fully viable, invasive condition.
(2) Studies designed to increase the percentage of schizont-infected cells present in the starting cell suspensions were proposed; the primary means for schizont enrichment would employ the use of density gradient ultracentrifugation.
(3) The percentage of schizont-infected cells present in vivo may be increased by inducing synchrony in the donor animals; studies were proposed to attempt synchrony induction, using massive intravenous doses of ring-stage parasitized cells.

Background:

Since the submission of the last annual report covering this Contract, several relevant reports concerning the study have appeared in the literature. The most significant effort has been recently published by Trager and Jensen (1), announcing their discovery that Plasmodium falciparum could be cultivated in vitro. These workers, using a stationary layer of cells and a modified medium RPMI 1640, have achieved near-physiological multiplication rates (Jensen, J.B., personal communication) at low parasitemias. Such a finding of course makes the development of a vaccine a much more likely possibility. In a related paper, Haynes et al. (2) have also reported successful cultivation of P. falciparum. These workers used a modification of Medium 199, another commercially available culture medium. The crucial conditions for culture appear to be a stationary layer of cells, a low $O_2$ tension, and an elevated $CO_2$ tension. If the parasitemia is permitted to rise much above 10%, the cultures rapidly

lose buffering capacity and die.

A recent report on the P. knowlesi model has proved relevant to the Contract studies. Dennis et al. (3) have used a sieve system to harvest free merozoites in vitro, using continuous perfusion. The schizont-infected cells are mostly retained above the surface of a polycarbonate sieve of small pore size, and as the schizonts rupture spontaneously, the merozoites are collected from beneath the sieve. The number of free merozoites harvestable in this fashion is quite large, and their viability is initially quite high; however, Dennis et al. found that after 39 min at 2°C, only about 1% of the free merozoites retained invasive ability. This report compares favorably to the earlier findings of Miller et al. (4), who found that at 37°C, free merozoites of P. knowlesi must reinvade host cells within 10-15 minutes in vitro.

Concerning the P. berghei model, Hamburger and Kreier (5, 6) and Kreier et al. (7) have reported several studies concerning the properties of free parasites isolated using continuous-flow ultrasonic lysis as a means for merozoite isolation. Mention will be made in a later section of these reports; they in essence show the free parasites to have properties which appear quantitatively different from intact parasitized cells in their response to antibody and in their antigenic properties, as assessed by polyacrilamide gel electrophoresis.

The Principal Investigator has published or submitted four articles in the past year, in conjunction with the findings of this Contract study (8,9,10,11). One of these reports deals with a means for the improvement of morphology on Giemsa-stained films of parasitized cells which have been washed or grown in vitro (8). The second article details the methods developed for schizont enrichment (objective #2 above), using density gradient ultracentrifugation employing Stractan II as gradient material (9). The third and fourth papers deal with the properties of the merozoites of P. berghei, harvested in a variety of different ways (objective #1 above) (10,11).

Approach to the Problem: Results and Discussion:

The mature schizonts of P. berghei may be selectively enriched using density gradient centrifugation using Stractan II as gradient forming material (9). The cells are found to be minimally injured by the gradient material, and may be substantially enriched using this method. However, the fractionation is not perfect, and the schizont-rich fraction will always be contaminated by cells at other stages of the infection, as well as non-parasitized cells. Even so, over 90% of all the schizonts present could normally be concentrated into a fraction containing only 20% of all the red cells present in the starting suspensions.

Attempts have been made to induce synchrony in mice by administering large doses of ring-stage parasites (the pellet from Stractan gradient centrifugation contains mostly normal cells and ring-stage parasitized cells). There was some evidence of synchrony early in the infection (soon after patency) in these mice, but the synchrony was rapidly lost as the infection progressed. It was concluded that no greater enrichment of schizonts could be achieved with these methods, than could be obtained by the gradient procedures for schizont enrichment.

A better degree of synchrony was found to be obtainable in mice by infecting them with parasitized cells sensitized with rabbit anti-mouse erythrocyte serum, and intravenous injection. Complement-mediated immune lysis then occurs in the recipient, and the resultant infections begin in a highly synchronous fashion. The basis for this phenomenon rests with the merozoites freed by immune lysis invading the host's cells, whereas all other intracellular stages, once rendered free in the recipients, do not contribute to the infection. The synchrony begins to disappear in these animals when the infection approaches 1-10% parasitemia. The loss of synchrony was found to be dependent on the total parasite burden, rather than the number of parasite generations which had passed after the infections were initiated. It appears that the parasites remain synchronous as long as there are susceptible polychromatophils available for parasitism. Once the number of polychromatophils becomes limiting, the parasites begin to invade normocytes, and concomitant with this shift in cell preference, a loss of synchrony occurs. The current hypothesis I am pursuing to explain this result is that the parasites show a more rapid course of development in normocytes than in polychromatophilic, or immature, erythrocytes.

In keeping with the objectives of the Contract, several means for merozoite isolation by artificial means have been evaluated (10). All were found to give excellent lysis of the host cells, although none ever gave 100% lysis. All methods tested, except for isotonic $NH_4Cl$, yielded free parasites having well-preserved morphology at the light microscope level. But none of the methods was found to yield merozoites possessing significant viability. These results applied both to invasive ability *in vitro* using a medium known to support invasion by this species (12), and *in vivo*. The methods found to give the best morphology and least host cell contamination were continuous-flow and multiple-burst sonication, followed by centrifugation at 1450 x $g_{min}$, 10 minutes. This centrifugation procedure has been stated to give free parasites of this species uncontaminated by intact host cells (5,6,7). This finding could not be repeated, even if several different volumes of host cell suspensions were

used, or if the value of 1450 x g was assumed to be maximum, minimum, or average. Subsequent experiments were done in vivo to evaluate the free parasites' infectivity. All infectivity of these preparations could be accounted for on the basis of the intact parasitized cells contaminating the preparations. An examination of other studies published in this area reveal only that of Walter (13) to have been conclusively performed using merozoites, and these studies were done in vivo. All other reports of P. berghei free parasites' infectivity [reviewed in (10)] are presumed to have been based on a small but finite number of intact parasitized cells.

These findings prompted the formulation of an hypothesis, that the free merozoites of P. berghei, once removed from the host cells, rapidly lose invasive ability, a property shared by P. knowlesi (3,4). The hypothesis was tested (11) using rabbit anti-red cell antibody-sensitized cells, and studying the properties of the free merozoites after lysis had occurred either in vitro or in vivo. The stability of the free merozoites was monitored, and the assays were all done in vivo to eliminate any artifacts of culture systems in vitro. Estimates of the true yield of viable merozoites harvestable under various conditions were also made in these studies.

Results of this approach revealed the artificial removal of merozoites to be followed by a rapid loss of viability. Within 15 minutes after lysis, all merozoites have lost invasive ability when held at 1-2$^{o}$C. Furthermore, this phenomenon is aggravated by washing the free parasites in an isotonic diluent not found to harm intact parasitized cells' morphology or infectivity. The washing (or simply holding on ice in diluent) of parasitized cells prior to sensitization was also found to greatly reduce the number of viable merozoites isolable following sensitization and lysis in vivo. Thus the merozoites of this species are extremely fragile creatures, and this fragility also applies to those within the mature schizont-infected cell. Furthermore, differential centrifugal enrichment of mature schizonts is probably unproductive, if the schizonts are then to be ruptured artificially. The indications are, however, that the damage done to the merozoites within the mature schizont may be reversible, if they are further incubated in medium at 37$^{o}$C. So differential centrifugation may be a valuable tool, if spontaneous schizont rupture is subsequently employed for merozoite isolation.

Using the findings of Walter (13) as a guide, the results of the present studies indicate that the merozoites within unwashed infected cells must be no further than 25 minutes away from spontaneous rupture, if they are to be viable when isolated artificially. The possibility must be considered that invasive ability is acquired in concert with spontaneous schizont rupture.

Several exploratory studies have been performed pursuant to the Contract this year. Included in these were studies regarding the question of virulence in the so-called "virulent line" of P. yoelii strain 17X (14). In my laboratory, this parasite has shown a tendency to spontaneously revert to an avirulent form when maintained in random bred Swiss mice. Several preliminary observations indicated that the reversion to avirulence was associated with a shift in cell preference on the part of the merozoites. Thus, efforts were made to experimentally shift the virulence of the parasite by infecting animals with parasitized cells differing markedly in their differential composition. No pattern of predictablility emerged from these studies; the reversion to avirulence appeared spontaneously and at random. It was concluded that this strain of parasite was particularly unstable in random bred Swiss mice.

Under the auspices of the Contract, one of my students has at my direction been performing preliminary studies regarding the nature of the erythrocyte receptors for parasite invasion. Using polyacrilamide gel electrophoresis employing sodium dodecyl sulfate, he has been comparing the banding patterns of erythrocyte ghosts prepared from suspensions of rat normocytes, rat reticulocytes, and mouse erythrocytes (normocytes and reticulocytes). Since the NYU-2 strain of P. berghei invades rat normocytes with great difficulty, but readily invades mouse cells of all ages, it was reasoned that these studies may give some clue as to the nature of the receptors involved. To date, these studies have given no results worthy of further pursuit.

Conclusions and Recommendations:

The use of artificial schizont rupture to yield free merozoites was anticipated to be a worthwhile and attainable goal when these studies were begun, based on other reports of successful methods employing P. berghei. Given the results of the past year, the following conclusions are now appropriate.

(1) Only a very small fraction of the merozoites contained in schizonts with 4 or more nuclei and residual body are sufficiently advanced to be capable of reinvading red cells after artificial schizont rupture using immune hemolysis.

(2) Washing a population of red cells with a non-toxic isotonic diluent selectively inactivates the late schizonts, so that the number of viable merozoites isolable by artificial means drops to insignificant levels.

(3) Once removed artificially, or using spontaneous schizont rupture [P. knowlesi (3,4)], the merozoites rapidly lose viability independent of the temperature at which they are maintained.

(4) Ultrastructural criteria used to date (15,16) do not

seem reliable in showing loss of invasive ability by the free merozoites of P. berghei.
(5) The loss of invasive ability occurs with rapidity at both low temperatures (1-2°C) and at physiological temperatures (35°C). Since washing seems to exacerbate the problem, it may be related to the diffusion-mediated loss of a critical material from the cell.

The renewal proposal submitted to the Command in Aug 76 has proposed studies designed to apply the findings of Trager and Jensen (1) to culture of P. berghei in vitro. The Principal Investigator feels that a complete commitment to the P. falciparum system for culture work would be inappropriate at this time, given the difficulties in performing studies in vivo with this species. It is felt that the rodent models offer several advantages for studying the properties of antigens harvested after culture in vitro, particularly regarding the studies of the antigens' stability, of antigenic variation, and physicochemical characterization of the protective antigens of mala[illegible]. These studies must be performed prior to the de[illegible]pment of an effective vaccine, and in the author's opin[illegible]not be done entirely in vitro. Studies are ther[illegible] planned to adapt the known methods supportive of P. falciparum growth to one or more species of rodent malaria, prior to beginning analyses of the properties of the protective antigens.

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